Plant Physiology and Biochemistry 76 (2014) 1–6

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Short communication

# Localization of polysaccharides in isolated and intact cuticles of eucalypt, poplar and pear leaves by enzyme-gold labelling

Paula Guzmán [a], Victoria Fernández [a,*], María Luisa García [b], Mohamed Khayet [c], Agustín Fernández [b], Luis Gil [a]

[a] Forest Genetics and Eco-physiology Research Group, School of Forest Engineering, Technical University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
[b] Electron Microscopy National Centre, Faculty of Chemistry, University Complutense of Madrid, Avda. Complutense s/n, 28040 Madrid, Spain
[c] Department of Applied Physics I, Faculty of Physics, University Complutense of Madrid, Avda. Complutense s/n, 28040 Madrid, Spain

ARTICLE INFO

*Article history:*
Received 3 December 2013
Accepted 30 December 2013
Available online 8 January 2014

*Keywords:*
Cellulose
Cuticle
Gold-labelling
Leaf
Pectins
Polysaccharides
Transmission electron microscopy

ABSTRACT

The presence and characteristics of cuticle polysaccharides have been demonstrated by staining and spectroscopic methods, but their location in the cuticle remains unclear. Furthermore, according to the prevailing model, polysaccharides are believed to be restricted to the cuticular layer and absent in the cuticle proper. With the aim of gaining insight into cuticular ultra-structure focussing on polysaccharides, cellulose and pectins have been identified and located in the transversal sections of isolated and intact adaxial leaf cuticles of *Eucalyptus globulus*, *Populus* × *canescens* and *Pyrus communis* by means of enzyme gold-labelling (Au-cellulase, EC 3.2.1.4, and -pectinase, EC 3.2.1.15) and transmission electron microscopy (TEM). The structure of the interface between the cuticle and the cell wall underneath was observed to influence the process of enzymatic isolation of leaf cuticles. Cellulose and pectins were detected for the first time in enzymatically isolated cuticles, sometimes appearing closely underneath the epicuticular wax layer. The location and presence of polysaccharides in intact and isolated leaf cuticles may have multiple implications, such as when estimating the bi-directional transport of substances between plant organs and the surrounding environment, or when interpreting organ ontogeny. The results are discussed within a plant ontological and ecophysiological context.



## 1. Introduction

The outer surface of most aerial organs of plants such as leaves, flowers, fruits and non-woody stems are covered with a composite, extracellular membrane, the cuticle, which plays a major physiological and structural role in the protection against multiple potential biotic and abiotic stress factors (Carpenter et al., 2007; Reina-Pinto and Yephremov, 2009). Due to the intimate relationship between the cuticle and the cell wall underneath, the cuticle may be considered a cutinized cell wall (Domínguez et al., 2011).

The concept of heterogeneity as a crucial component of cell wall structure and function (Marga et al., 2003; Burton et al., 2010) can be extended to the cuticle. From a chemical point of view, the cuticle is mainly composed of a cutin and/or cutan polymer matrix, waxes both embedded in (intracuticular) and deposited onto (epicuticular) the outer surface of such matrix, polysaccharides and phenolics (Domínguez et al., 2011; Bernal et al., 2013).

According to the prevailing model (von Mohl, 1847), the cuticle consists of two different layers, the cuticle proper (CP) as the outermost region and the subjacent cuticular layer (CL). An epicuticular wax (EW) layer covers the CP and is in direct contact with the environment (Jeffree, 2006). The CP, which is by definition free of polysaccharides, is the first cuticular region to appear during cuticle ontogeny, assembled to the primary cell wall by a pectic layer. The CL contains polysaccharides steaming from the epidermal cell wall and appears later during cell wall development (Jeffree, 2006).

Cellulose, hemicelluloses and pectins have been grossly isolated from tomato fruit cuticles, and they have been observed to play an important role on cuticle rheological properties (López-Casado et al., 2007). The presence and characteristics of polysaccharides in leaf and fruit isolated cuticles examined as intact tissues or after cutin depolymerisation, have been analysed by different spectroscopic methods [e.g., (Domínguez and Heredia, 1999; Johnson et al., 2007; López-Casado et al., 2007)]. Although it was found that

* Corresponding author. Tel.: +34 913367113.
*E-mail addresses:* v.fernandez@upm.es, victoria.fernandez@gmx.de (V. Fernández).

0981-9428/$ – see front matter © 2014 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.plaphy.2013.12.023

cellulose fibres are mainly randomly oriented in cuticles of mature and ripe tomato fruits and *Agave Americana* young leaves (Domínguez and Heredia, 1999; López-Casado et al., 2007), the position and distinction between groups of polysaccharides cannot be ascertained by such analytical techniques. On the other hand, stains like osmium tetroxide-uranyl acetate-lead citrate combination (Wattendorff and Holloway, 1982), calcofluor white (López-Casado et al., 2007), ruthenium red (Norris and Bukovac, 1968), periodic acid-thiosemicarbazide-silver proteinate tests (Wattendorff and Holloway, 1982; Gouret et al., 1993) or chlor-zinc-iodine (Matas et al., 2004) have been used for detecting polysaccharides in cross-sections of isolated leaf and fruit cuticles by electron, optical and fluorescence microscopy. Nevertheless, the lack of specificity of the dyes, the potential degree of impregnation of polysaccharides with chiefly lipidic cuticular components, and the restrictions to observe stained tissues at higher magnifications by optical and fluorescence microscopy, pose an obstacle for elucidating the nature and location of polysaccharides in the cuticle.

Most of such constraints can be overcome by using gold (Au)-labelling techniques and transmission electron microscopy (TEM). Gold-enzyme labelling has proved to be a reliable tool for the identification and localization of selected polysaccharides in plant cell walls [e.g., (Berg et al., 1988; Ferguson et al., 1998)]. Working with spruce leaf transversal sections and Au-enzymatic labelling, Tenberge (1992) identified cellulose and hemicelluloses cuticle by, but failed to detect pectins.

In light of the current lack of information about the location of polysaccharides in leaf cuticles, we carried out enzymatic Au-labelling experiments to identify and locate cellulose and pectins in the adaxial leaf cuticle of three model plant species. European pear was selected since its leaf cuticle has been previously described (Norris and Bukovac, 1968). The leaf cuticle of grey poplar has been analysed since it is a model species used for cuticular permeability studies (Schreiber and Schönherr, 2009). The blue-gum eucalypt leaf has been examined due to its evergreen nature, markedly different eco-physiological habitat and commercial significance in forestry.

## 2. Materials and methods

### 2.1. Plant material

Fully expanded leaves of *Eucalyptus globulus* Labill., *Populus* × *canescens* (Ait.) Sm. (*P. alba* L. × *P. tremula* L.) and *Pyrus communis* L. var. Blanca de Aranjuez, hereinafter referred to as eucalypt, poplar and pear, were selected for experimental purposes. Juvenile eucalypt leaves were collected from 1.5-year-old seedlings growing in the Forest Engineering School Arboretum (Technical University of Madrid, Spain). Poplar leaves were obtained from trees previously identified with molecular analyses grown in Losana (Soria, Spain; R Sierra, Personal communication). Pear leaves were collected from trees grown in the Royal Botanic Gardens of Madrid (CSIC, Spain). All the leaves were collected from medium size shoots during the summer.

Prior to isolating the cuticles enzymatically, leaf midveins and margins were removed with a scalpel. The enzymatic solution contained 2% cellulase (EC 3.2.1.4, extracted from *Trichoderma reesei*, 10–20% w/v; Celluclast®, Novozymes, Bagsvared, Denmark), 2% pectinase (EC 3.2.1.15, from *Aspergillus aculeatus*, 10% w/v; Pectinex Ultra SP-L, Novozymes) plus 1% polyvinylpyrrolidone (Sigma–Aldrich, Munich, Germany) and 2 mM sodium azide [(Orgell, 1955); with some modifications]. The pH was adjusted to 5.0 by adding sodium citrate. Eucalypt and pear leaves were maintained in the solution for one month, until the cuticle appeared to be fully separated from the underlying tissue, while poplar leaves remained in solution from one and a half to two months. Leaf tissues were digested at room temperature (23–25 °C), solutions were changed every two weeks and they were manually shaken at frequent time intervals. After the extraction period, clean intact adaxial cuticles were selected, thoroughly washed in deionized water, air-dried and stored for microscopic examination.

### 2.2. Tissue fixation and embedding

Enzymatically digested adaxial cuticles and fresh leaves were cut into about 4 $mm^2$ pieces and subsequently fixed and embedded in LR-White resin, which is commonly used for immunocytochemistry and in relation to colloidal Au-labelling (Robards and Wilson, 1993). Samples were fixed in 1% glutaraldehyde-4% paraformaldehyde (both from Electron Microscopy Sciences (EMS), Hatfield, USA) for 4 h at 4 °C, rinsed in ice-cold phosphate buffer, pH 7.2, four times within a period of 6 h and left overnight. They were then dehydrated in an ethanol series of 30, 50, 70, 80, 90, 95 and 100% (x 2, 15 min each concentration) and embedded in ethanol-LR-White resin (London Resin Company, London, UK) solutions (3:1, 1 h; 1:1; 1 h; 1:3; 2 h (v:v)) and in pure resin overnight. The embedding protocol was performed on ice. Final embedding was done in capsules which were subsequently incubated at 50 °C for 3 d.

### 2.3. Preparation of the gold-enzyme complex and tissue labelling

Cellulose and pectins were detected in plant tissues by enzymatic Au-labelling. Since our goal was to examine the location of such polysaccharides in isolated cuticles, but not determining their specific epitopes, the use of antibodies was not considered in this study. Hence, the commercial mixtures Celluclast and Pectinex, conjugated with 10 nm citrate-coated Au nanoparticles (~0.01%; Sigma–Aldrich) were used. The Au-conjugated enzymes were prepared shortly before tissue labelling according to the methods described by Berg et al. (1988) and Ferguson et al. (1998), with some modifications.

Briefly, 5 ml of the colloidal Au solution were adjusted to pH 4.5 by adding sodium hydroxide. Concerning cellulase, 1 ml of the Au solution was mixed with 20 μl of a 0.2% solution of the enzymatic mixture. A 2.5:1 colloidal Au:enzyme ratio was selected for preventing the flocculation of the Au colloid in the final solution (de Roe et al., 1987). After 5 min stirring of the Au-enzyme solution, 100 μl of 1% polyethylene glycol (PEG, Sigma–Aldrich) were added in order to improve the stability of the Au-enzyme complex and to minimize non-specific enzyme adsorption to the tissues. The mixture was then centrifuged at 20,000 *g* for 1 h at room temperature. The resulting product had clear, supernatant and pink aqueous phases and a hard pink pellet. The clear aqueous phase was discarded and the pink one resuspended in 1 ml of 0.05 M citrate buffer, pH 4.5, with 0.1% PEG. Centrifugation and resuspension were repeated and the final Au-cellulase complex was stored at 4 °C for further use. The same procedure was followed for preparing the Au-pectinase complex.

The pH value of the colloidal Au-enzymatic solution was selected taking into consideration the isoelectric points (pI) of the enzymes (Essa et al., 2007). The most abundant enzyme produced by *T. reesei* is cellobiohydrolase I representing up to 60% of the total protein content secreted by the fungus (Rosgaard, 2007). The pI of this enzyme is 4.5–5.2 (lowest at 3.5; (Xu et al., 2011)). On the other hand, Pectinex contains several enzymes with pectolytic activities including polygalacturonase (product datasheet). As homogalacturonan may represent about 65% of pectins (Mohnen, 2008) and the main enzyme degrading its backbone is polygalacturonase, the pI of this enzyme (from *A. aculeatus*) was considered, being in the range 4.2–4.6 (Cho et al., 2001). Therefore, all the solutions